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Evaluating the value of soluble form of toll-like receptors 2 and 4 as a potential biomarker in multiple sclerosis

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Abstract

Available studies have suggested significance of soluble toll like receptor 2 and 4 (sTLR2, and 4) as endogenous first-line blockers of TLR signalling pathway. We hypothesized that sTLR2 and 4 may have the potential to be used as biomarkers in multiple sclerosis (MS) patients during relapses. To address this issue, serum and urine samples from MS patients during relapse and remission and from healthy individuals (H.C) were analysed with ELISA to determine the level of sTLR2 and 4. In serum samples, the concentration of sTLR2 was significantly higher in relapse patients compared with H.C (p value=0.025). However, no significant differences were observed in serum sTLR2 levels between relapse and remission states, or between remission and H.C. Urine sTLR2 levels in MS patients showed no significant differences between relapse, remission and H.C groups. Additionally, there were no significant differences in the levels of sTLR4 between relapse, remission and H.C groups both in serum and urine samples. The higher levels of sTLR2 may reflect an active state of the immune system during MS relapse. A patient with high sTLR2 value in urine was positive in the dipstick test, indicating a possible correlation between sTLR2 levels and sign of urinary tract infections. However, for urine sTLR4 levels, no correlations with dipstick results were observed. In conclusion, these results showed the presence of sTLR2 and 4 in MS patients' serum and urine, but analysing large number of patients is essential to confirm the value of sTLRs as potential biomarker of disease activity.

Keywords: Multiple Sclerosis (MS); Soluble Toll-Like Receptor; Biomarkers; Disease Relapse and Disease Remission

1. Introduction

1.1. Toll like Receptors

Before Toll-like receptors (TLRs) were discovered, innate immunity was considered as a relatively unsophisticated part of the immune system. Innate immunity is considered as the first line of host defence against infections and enables the mammalian immune system in discriminating between "self" and "non-self" antigens [1]. TLRs have a crucial role in the innate immune recognition of different types of pathogens including viruses, fungi and bacteria and differentiating them from self-antigens. TLRs are the first class of pattern recognition receptors (PRRs) that play an important role in recognizing both the foreign or pathogen derived components, known as pathogen associated molecular patterns (PAMPs) which are microbial structures, and also endogenous or host-derived molecules known as danger-associated molecular patterns (DAMPs) [2]. So far, there have been up to 12 functional TLRs identified in humans and mice, including TLR2 and TLR4 which have been conserved in both species [2]. Ten TLRs have been encoded in the human genome that respond and recognize PAMPs [3].

Both TLR2 and TLR4 are cell surface receptor of the TLR family. TLR2 is ubiquitously expressed by immune (both innate and adaptive) cells, endothelial cells, epithelial cells and nervous system cells [4, 5]. TLR2 is also known to preferentially

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expressed by Tregs than other T cells and forms heterodimers with either TLR1 or TLR6 [6]. TLR4 can be expressed on myeloid DC, monocytes, and macrophages, but also on T and B lymphocytes [7]. It recognizes cell-wall components such as lipoteichoic acid, peptidoglycan and lipoprotein from gram-positive bacteria, zymosan from yeast cell wall, and lipoarabinomannan from mycobacteria; while TLR4 exclusively recognizes lipopolysaccharide (LPS) from gram-negative bacteria, which is a potent activator of the immune system [7]. Besides, both TLR2 and TLR4 were reported to have a wide range of endogenous ligands which could be categorised as released intracellular proteins, extra cellular matrix components, oxidatively modified lipids, and other soluble mediators but the emerging evidence claims that many of these molecules may be more accurately described as PAMP-sensitizing molecules (PSMs), or PAMP-binding molecules (PBMs) rather than genuine ligands of TLR2 or TLR4 [1] . Thus, it could be assumed that inappropriate or prolonged activation of these receptors with endogenous ligands even in the absence of any infectious stimuli, might contribute to the development of chronic inflammation and autoimmunity.

TLRs are type I transmembrane glycoproteins with a tri-modular structure which include extracellular leucine rich repeats (LRR) responsible for PAMP binding, a transmembrane domain and the intracellular Toll/interleukin (IL)-1 receptor (TIR) domain which interacts with adaptor proteins and mediates the downstream signalling [8] .The Toll/IL-1R (TIR) domain in TLRs is known as a conserved cytoplasmic domain [9]. The TIR domains have been characterized by the presence of three boxes (known as boxes 1, 2 and 3), which are highly homologous regions [9]. After TLRs encounter PAMPs, it activates signalling cascades via TIR domain-containing adaptors, like myeloid differentiation primary response protein 88 (MyD88)-dependent activation of nuclear factor-κB (NF-κB) and TIRAP [3, 10]. MyD88 is common for all the TLRs, while TIRAP is involved specifically in TLR2 and TLR4- mediated signalling pathways (Figure 1) [10]. TLR4 uses both MyD88-dependent and MyD88-independent signalling pathway [11]. In addition to the common MyD88-dependent pathway, this independent pathway leads to the activation of IRF-3 and induction of IFN-β [10]. TLRs can recognise PAMPs either alone or in connection with co-receptors [12]. For example, TLR4 requires an additional protein called myeloid differentiation factor2 (MD-2), also known as (LY96) to respond to LPS efficiently [3, 13]. On the cell surface, TLR4 with MD2 form a complex, and which together function as the main LPS-binding component [2]. Additional proteins such as cluster of differentiation CD14 and LPS-binding protein (LBP) are also thought to be involved in LPS binding [2]. CD14 is the co-receptor required for both TLR2 and TLR4 signalling that enhance the sensitization by the ligands. LBP is a soluble plasma protein which binds LPS; and CD14 is a glycosylphosphatidylinositol-linked, leucine-rich repeat which binds LBP and delivers LPS-LBP to the TLR4-MD2 complex [2]. After the activation of both TLR2 and TLR4 by their ligand and with the association of the accessory molecules on the cell surface, they will induce the intracellular signalling cascade leading to the NF-κB activation and the production of pro-inflammatory cytokines [11]. Besides LPS, TLR4 also recognizes a variety of endogenous proteins such as heat shock proteins, beta-defensins, and low-density lipoprotein, hyaluronic acid, a high mobility group box chromosomal protein 1 (HMGB1), heparan sulphate, and fibrinogen [1]. Heat shock protein (HSP60), HSP70, HMGB1, cardiac myosin, biglycan, hyaluronan, versican, serum amyloid A (SAA), and amyloids are some of the endogenous ligands that can interact with TLR2 in relation to different cell types [1].

sTLRs can be detected in human body fluids, including amniotic fluid, saliva, pleural fluid, plasma, and breast milk [15, 16]. sTLRs have been considered as a first-line blockade in TLRs signalling, extracellular domains of TLR2 and 4 have been actively released to behave as a decoy receptors [16]. At least six sTLR2 polypeptides ranging from 25-83 kDa have been identified in human breast milk, plasma and the monocyte culture supernatant [17]. Soluble TLR2 and TLR4 have been reported to be capable of ligand (DAMPs and PAMPs) binding, however; they do not transmit the signal downstream [16]. There could be two possible ways in which sTLRs dampen the inflammatory signal: unavailability of the ligands to bind with the cell surface receptor or decrease in the expression of the receptors on the cell surface. Soluble TLR4 still retains the capacity to bind its ligands, but inhibits LPS binding by cell membrane TLR4 and thus prevents TLR4-mediated inflammatory signalling [15, 18]. Ectodomain shedding has been documented well as a mechanism for the generation of soluble form of the cytokine and chemokine receptors, and could provide a more direct way of inhibiting activation of individual TLRs [19]. While there is evidence for sTLR4 generation by alternative splicing (by post-transcriptional modifications), sTLR2 can be produced by post-translational modifications, such as protease cleavage [18, 20].

1.2. Multiple sclerosis

Multiple sclerosis (MS) is a chronic, inflammatory, immune-mediated neurological disease of the central nervous system (CNS), including the brain, spinal cord, and the optic nerve [21, 22]. At least 2.5 million people are affected by MS worldwide and there are more than 100,000 people living with MS in the UK [22]. MS is destroying the axons and the myelin to varying degrees, after attacking the myelinated axons in the CNS [21]. The severity and timing of each attack are unpredictable and can be devastating, leaving the patient severely disabled, resulting in serious physical disability in young adults, especially women (2-3:1 female to male ratio) [22, 23]. Eventually, after many years of disease, gradual

accumulation of disability leads to the permanent use of a wheelchair for up to 50% of the patients [22]. Relapsing-remitting MS (RRMS) is characterized initially by episodes of reversible neurological deficits known as relapses, which are often followed by progressive neurological deterioration over time known as secondary progressive MS (SPMS) [21]. Clinically, MS relapse is defined as a sudden and transient neurological deficits which lasts for at least 24 hours and are not just due to an infection or a rise in the body temperature [24]. Clinical symptoms of MS include problems with speech, bladder control, and vision, also weakness, numbness, and loss of motor coordination [23]. The most common form of this disease is RRMS, affecting about 85% of MS patients [21]. It is marked relapses (exacerbations, flare ups) of symptoms followed by periods in which symptoms disappear or improve, called remission [21].

1.3. Biomarker and Immunotherapy

A biomarker is a characteristic that is measured objectively and estimated as a signal of normal biological mechanism, pharmacologic responses, or pathogenic processes, to a therapeutic intervention [24]. Currently, there are several biomarkers under investigation for MS. Biomarkers are being sought actively for the early diagnosis and the identification of individuals at risk of developing MS, the monitoring of disease progression, the prediction of disease course, the selection of specific treatments, the staging of MS patients, and the monitoring of response to therapy and the evaluation of novel therapeutics [24].

Inflammatory activity is heightened during relapses in MS patients but the exact molecular trigger is not clearly known to date. There is a growing need for finding a biomarker of inflammatory activity during MS relapses, ideally one that precedes the onset of exacerbation. Endogenous ligands of both TLR2 and TLR4 such as HMGB1 have been found to be increased in expression in the CSF mononuclear cells of MS patients compared to healthy controls [25]. In the peripheral blood mononuclear cells (PBMCs) from MS patients also found that TLR2, and TLR4 expression were upregulated compared with healthy controls [26]. Soluble form of TLR4 is encoded by alternatively spliced TLR4 mRNA that still retains the capacity to bind its ligands, but inhibits LPS binding by cell membrane TLR4 and thus prevent TLR4 mediated inflammatory signalling [18]. sTLR4 and MD-2 complex probably blocks the interaction between membrane-bound TLR4 and its ligand [13]. Soluble TLR2 and TLR4 are hypothesized to be released in circulation during inflammatory insults and dampen inflammation. Furthermore, they have been reported to be released in vitro by stimulated PBMC and also in vivo during experimental human endotoxemia [12]. Notably, they also found that sTLR2, and sTLR4 concentration was significantly higher in circulation (plasma) of patients with infection compared with patients with non-infectious inflammation and again both of these soluble form of TLRs level was higher in patients having bacterial infection compared to patients having viral infection [12]. Thus both sTLR2, and sTLR4 could play an important role in the modulation of inflammation during infections. It is hypothesized that sTLRs may have the potential to be used as diagnostic biomarkers during relapses in MS patients. The soluble decoy receptors that have been induced by various stimuli provide negative regulatory mechanisms for their respective receptor interactions, and for cytokines and chemokines, therefore not surprising that sTLRs that might be effective in blocking TLR signalling [27]. There are some studies highlighting the association of TLR2 and TLR4 with MS [26]. However, no previous studies have reported the role of sTLR2 or sTLR4 in MS. Hence, in this project, we measured the level of sTLR2 and sTLR4 in serum and urine of MS patients and compared them with healthy controls. There is at least one paper that reported the detection soluble form of TLR4 released in the urine as a biomarker of disease activity in patients with acute kidney injury who develop cirrhosis [28]. They found that sTLR4 in urine was significantly higher in patients with renal dysfunction associated with infection/inflammation [28]. For this study, serum and urine samples from RRMS patients were screened for sTLR2 and sTLR4 levels and compared with healthy controls.

2. Material and methods

2.1. Sample Collection and Storage

Serum and urine samples were collected from patients during relapse and remission state. Serum and urine samples were also obtained from healthy individuals. A serum separator tube (SST) was used for collection of serum from the patients, and then samples were left to clot for 30minutes at room temperature before centrifugation for 10 minutes at 448 x g. After that the serum was aliquoted in Eppendorf tubes (ET, 400µL/aliquot), the samples were processed inside the safety cabinet to prevent any contamination. After that samples were stored at -80°C. Repeated freeze-thaw cycles were avoided. Midstream urine was collected aseptically and voided directly into a sterile container. Then the samples were tested for signs of infection with a urine dip-stick test (Combur-Test® strips (Cobas-Roche; No. 4510062171) and aliquoted (400µL/aliquot) and stored at -80°C).

2.2. Urine Dip-stick Analysis

Urine dip-stick test with Combur-Test® strips (Cobas-Roche; No. 4510062171) were performed to detect the presence of Leukocytes, Nitrites, Protein, and Erythrocytes in urine samples as a sign of urinary tract infections (UTI) which could reflect mild inflammation to severe infections.

2.3. Calculation of the ELISA results

Average optical density (O.D) of duplicate readings for each standard, control and sample were calculated and from these the average O.D of the zero standard or blank was then subtracted. A standard curve was created for each experiment, by plotting sTLR2 or sTLR4 standard concentrations on the x-axis and absorbance or mean O.D on the y-axis. Then, from the standard curve, the unknown concentrations for the samples were determined.

2.4. Statistical analysis

Soluble forms of TLR2, and TLR4 were evaluated by using Microsoft® Excel 2010 (Microsoft Co) and GraphPad Prism V-7.01. Analysis of sTLR2 concentration in urine and serum samples from multiple sclerosis patients compared with healthy controls was done using the Dunn's multiple comparisons test. The difference between paired relapse and remission samples was measured using Wilcoxon matched-pairs signed rank test. Statistically significant data were identified as having a $p<0.05$.

3. Results

3.1. The level of sTLR2 in serum of MS patients is significantly higher during relapse compared to the healthy controls

Serum samples from 6 patients during relapse and remission states and 3 healthy individuals were tested to detect sTLR2 levels, as previously described in section 2.4 using human TLR2 Quantikine® ELISA kit (R and D systems, Catalogue Number DTLR20).

Soluble form of TLR2 concentration was found to be significantly higher in patients during relapse (0.880ng/ml) compared to healthy control (0.456 ng/ml) (Table1). The Dunn's multiple comparisons test gave a p value of 0.025. However, there was no significant difference between relapse and remission states (p value>0.999), and between remission state and healthy control (p value=0.105) (Figure 2A).

Table 1 Level of sTLR2 in serum and urine (ng/ml) measured by sandwich ELISA in MS patients and healthy controls (H.C)

Serial	Serum			Urine		
	Relapse	Remission	H.C	Relapse	Remission	H.C
1	0.738	0.658	0.485	0.185	0.00	0.00
2	0.619	0.831	0.504	4.116	5.445	1.189
3	1.054	0.565	0.381	0.729	1.053	1.500
4	0.954	0.931		0.746	0.472	0.496
5	0.654	0.527		0.428	0.539	
6	1.261	1.081				
Average	0.880	0.765	0.456	1.241	1.502	0.796
S.D	0.253	0.219	0.066	1.624	2.235	0.676

The concentration of sTLR2 in individual patients at different time points in relapse and remission shows a trend where in most of the patients (5 out of 6), sTLR2 values decreased in remission (0.765) than in relapse (0.880) patients; except for one patient, in which sTLR2 concentration was increased in the remission compared to relapse (Table 1). Among the 6 paired samples, 5 patients had higher sTLR2 values in relapse, 1 patient had higher value during remission.

Wilcoxon matched-pairs signed rank test showed no significant difference between paired relapse and remission samples (P value= 0.312) (Figure 2B).

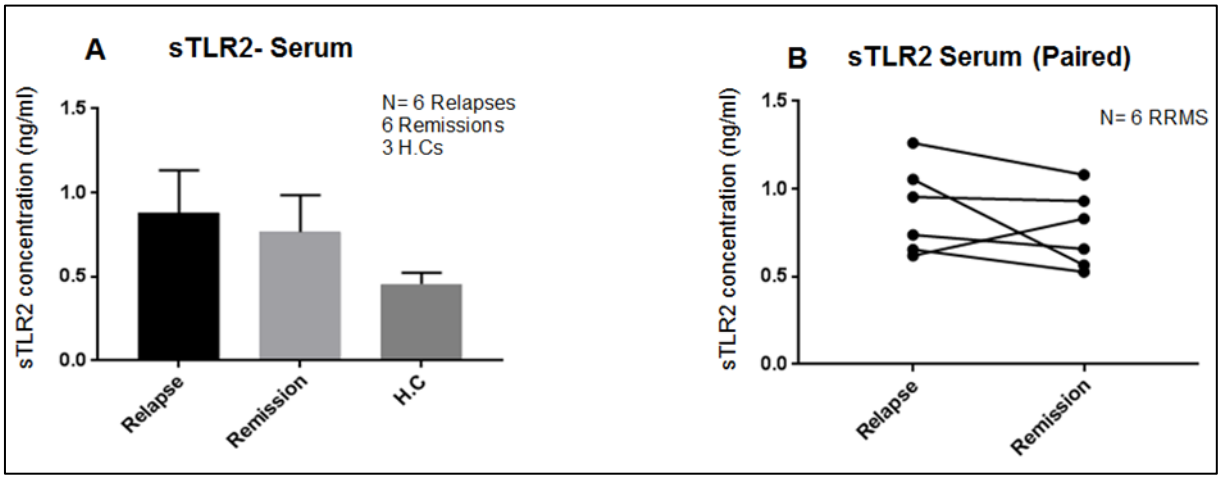

Figure 2 sTLR2 level (ng/ml) in serum of relapsing-remitting MS (RRMS) patients and healthy controls

Serum samples from six patients both during relapse and remission state and three healthy individuals were screened to detect sTLR2 levels using ELISA $p<0.05$ was considered as significant **(A)** The Dunn's multiple comparisons test showed, the concentration of sTLR2 significantly increased in relapse patients compare to healthy individuals ($p=0.025$). However, there was no significant difference between relapse and remission patients ($p>0.999$), and also between remission patients and healthy control ($p=0.105$). The results are presented as mean and standard deviations. **(B)** Concentration of sTLR2 was lower in all serum samples from remission than in relapse, with the exception of one patient where sTLR2 concentration was higher in remission. The result was analysed using a Wilcoxon matched-pairs signed rank test ($p=0.312$).

3.2. The level of sTLR2 in urine of MS patients is not significantly different from healthy controls

Frozen urine samples collected from 5 patients during relapse and remission phases, and 4 healthy individuals were analysed for sTLR2 levels by ELISA Using human TLR2 Quantikine® ELISA kit. The concentration of sTLR2 in remission (2.235 ng/ml), relapse (1.624 ng/ml), and in healthy control (0.676 ng/ml). We could not detect significant changes in the levels of sTLR2 in urine samples of MS patients between relapse and remission states (p value=0.968), and neither found a significant difference between the patient's groups and healthy controls (p values were 0.920 between relapse and healthy controls, and 0.813 between remission and healthy controls). The result was assessed using Dunn's multiple comparisons test.

Among the 5 paired samples, 3 patients had higher sTLR2 values in remission, 2 patient had higher value during relapse. Wilcoxon matched-pairs signed rank test showed no significant difference in sTLR2 levels between paired relapse and remission samples (p value=0.625, Fig. 3B). The standard deviations were high between groups. The values were very close in relapse and remission for the same patient except in one patient, which showed to have exceptionally higher values both in relapse (4.116 ng/ml) and remission (5.445 ng/ml) with a sharp increase in remission (second urine sample in Table 1). Dipstick test have been performed to test for the presence of Leukocytes, Nitrites, Protein, and Erythrocytes as a sign of infection and to see if there is any correlation between infection status and levels of sTLR2 in urine. It was noted that the second urine sample that gave a surprisingly higher values of sTLR2 was highly positive for all the four parameters in the dip-stick test that could account for the sign of an ongoing urinary tract infection (Table 2).

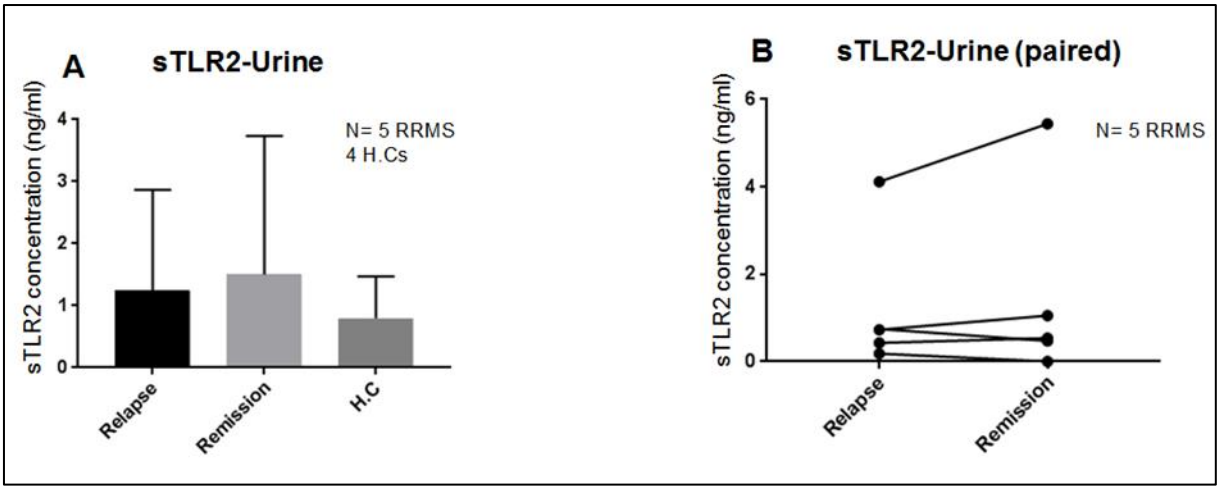

Figure 3 sTLR2 level (ng/ml) in urine of relapsing-remitting MS (RRMS) patients and healthy controls

Urine samples were collected from 5 relapse-remitting patients and compared to samples from 4 healthy individuals. Soluble TLR2 levels were detected using ELISA assay. **(A)** No significant amount of sTLR2 was detected in patients during relapse and remitting when compared to healthy controls. The result was assessed by a Dunn's multiple comparisons test (p value for relapse-remission = 0.968, relapse-healthy control= 0.920, remission-healthy control= o.813). The results are presented as mean and standard deviations. **(B)** Wilcoxon matched-pairs signed rank test, there was no significant difference between paired relapse and remission samples (*P* value = 0.625)**.**

Table 2 Dipstick test results for the urine samples used for sTLR2 measurement

Serial	**Relapse**				**Remission**			
	Leu	**Nit**	**Prot**	**Eryt**	**Leu**	**Nit**	**Prot**	**Eryt**
1	-	-	-	-	-	-	-	-
2	3+	2+	2+	2+	3+	pos	1+	-
3	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-
5	2+	-	-	-	-	1+	-	1+

Note: Leu= Leukocytes, Nit= Nitrite, Prot= Protein, Eryt= Erythrocytes

3.3. The level of sTLR4 is not significantly different in the in serum of MS patients compared to the healthy controls

Serum was collected from 13 patients during relapse and 11 patients in remission states, and from 7 healthy individuals (Table 3). Using human TLR4 ELISA kit (Cloud-Clone Corp., Houston, TX, USA). Soluble form of TLR4 concentration in relapse (1.539 ng/ml), remission (1.413 ng/ml), and in H.C (0.262 ng/ml). There was no significant difference in the concentration of sTLR4 detect in relapse, remission, and in healthy control (*p* value for Relapse vs Remission =0.001, relapse vs healthy control 0.493, remission vs healthy control 0.581. Fig. 4A) the result was assessed by Dunn's multiple comparisons test. Analysing the result with Wilcoxon matched-pairs signed rank test also did not show any significant difference of sTLR4 levels between the relapse and remission samples (*p* = 0.769, Fig. 4B). Among the 11 paired samples, 6 patients had higher sTLR4 values in relapse, 4 patients had higher value during remission and in 1 patient it was below detectable range (considered as 0 ng/ml) for both relapse and remission. However, overall analysis showed no significant differences between the paired relapse and remission samples (*p* = 0.769, Fig. 5B)

Table 3 Level of sTLR4 in serum and urine (ng/ml) measured by sandwich ELISA in MS patients and healthy controls (H.C)

Serial	Serum			Urine		
	Relapse	Remission	H.C	Relapse	Remission	H.C
1	0.00	0.00	0.00	0.00	1.264	0.00
2	0.233	0.226	0.00	0.797	1.448	0.00
3	0.00	0.595	0.294	0.097	0.00	0.203
4	7.167	9.973	0.00	0.00	0.00	0.082
5	0.719	0.00	0.271	0.00	0.00	0.286
6	1.888	1.395	0.00	0.00	0.158	0.090
7	6.789	0.685	1.269	0.00	0.044	0.00
8	1.464	0.513		0.00	0.00	0.00
9	0.00	1.039		0.00	0.00	
10	0.605	0.296		0.00	0.273	
11	0.662	0.822		0.00		
12	0.00			0.00		
13	0.49					
Average	1.540	1.413	0.262	0.074	0.319	0.083
S.D	2.482	2.871	0.464	0.229	0.556	0.109

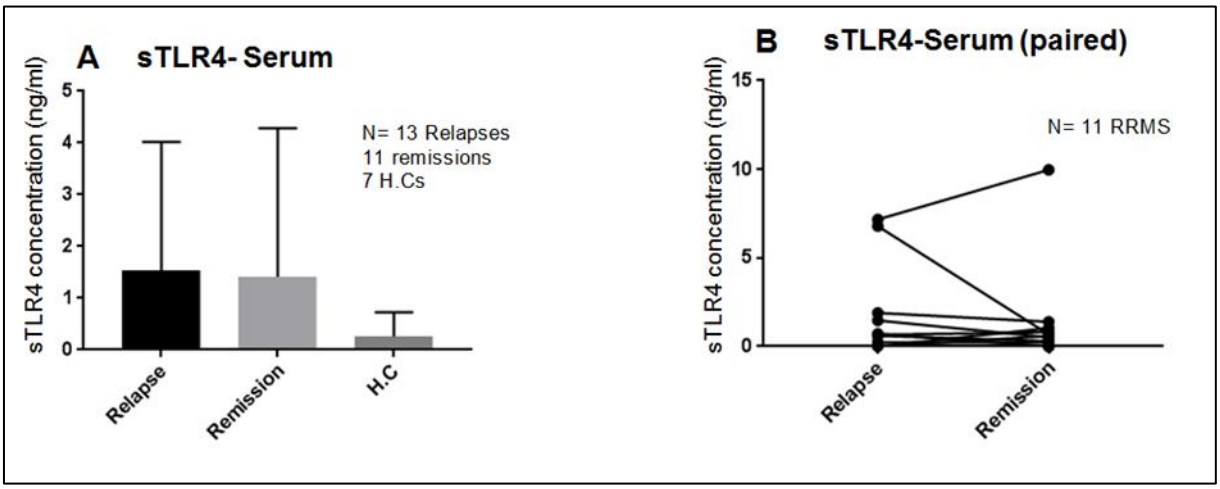

Figure 4 sTLR4 levels (ng/ml) in serum of relapsing-remitting MS (RRMS) patients and healthy controls

Serum was collected from relapses, remissions patients and from healthy individuals. Soluble TLR4 levels were detected using ELISA assay. **(A)** Using Dunn's multiple comparisons test, there was no significant differences in sTLR4 concentration in patients during relapse, remission, and in healthy control (*p* value for relapse-remission 0.001, relapse-healthy control 0.493, remission-healthy control 0.581. Fig. 4A). The results are presented as mean and standard deviations. **(B)** Concentrations of sTLR4 in relapse patients were paired with those from remission patients. The result was analysed with Wilcoxon matched-pairs signed rank test which showed no significant difference (p=0.769).

3.4. The level of sTLR4 is not significantly different in the urine of MS patients compared to the healthy controls

Concentration of sTLR4 was detected in urine samples collected from 12 patients during relapse, 10 patients during remission states, and 8 samples from healthy individuals (Table 3). The samples were then analysed using human TLR4 ELISA kit (Cloud-Clone Corp., Houston, TX). Soluble form of TLR4 concentration in relapse (0.074 ng/ml), remission (0.319 ng/ml), and in healthy control (0.083 ng/ml). There were no significant differences found in sTLR4 concentration in relapse, remission, and in healthy control (p value: relapse vs remission =0.308, relapse vs healthy control =0.649, remission vs healthy control =0.999 Fig. 5A) the result was assessed using Dunn's multiple comparisons test. Among the samples, 9 patients had paired samples (relapse and remission sample for the same patient). Among the 9 paired samples, increased sTLR4 values in 4 patients in remission, 1 patient had higher value during relapse and in 4 patients it was below detectable range (considered as 0ng/ml) for both relapse and remission. However, overall analysis showed no significant differences between the paired relapse and remission samples (p value= 0.187, Fig. 5B) the result was assessed by Wilcoxon matched-pairs signed rank test.

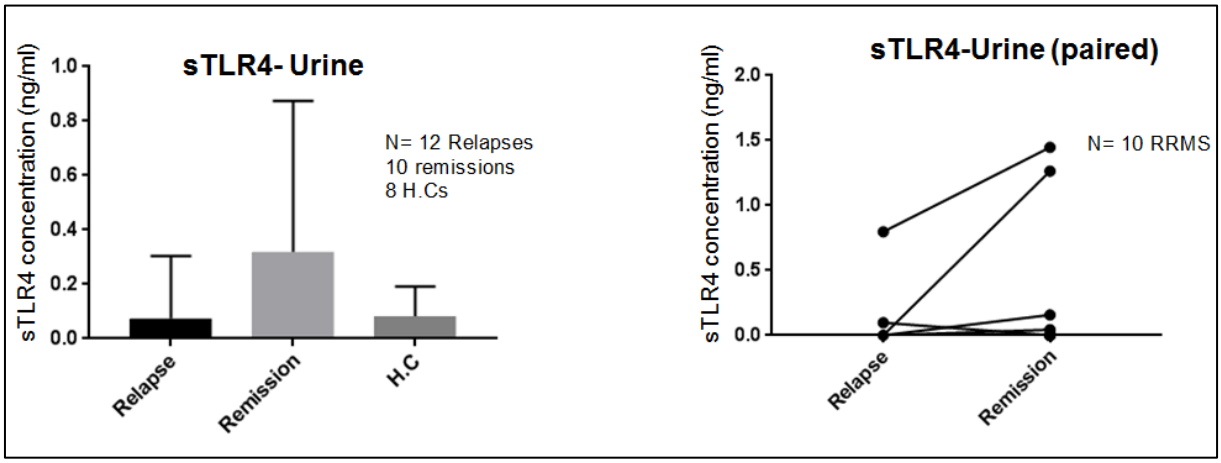

Figure 5 sTLR4 levels (ng/ml) in urine of relapsing-remitting MS (RRMS) patients and healthy controls

Urine was collected from 12 patients during relapse, 10 patients during remission states, and 8 samples from healthy individuals. **(A)** Using Dunn's multiple comparisons test, there were no significant differences in sTLR4 concentration in patients during relapse-remitting (p=0.308), relapse-healthy control (p=0.649), remission- healthy control (p>0.999). The results are presented as mean and standard deviations. **(B)** Concentration of sTLR4 was similar in all urine samples. With the exception of one patient sample, where sTLR4 concentration was higher in remission, and another sample where sTLR4 was higher in relapse. The result was analysed using a Wilcoxon matched-pairs signed rank test (p= 0.187).

Table 4 Dipstick test results for the urine samples used for sTLR4 measurement

Serial	Relapse				Remission			
	Leu	Nit	Prot	Eryt	Leu	Nit	Prot	Eryt
1	-	-	-	-	-	-	-	-
2	3+	3+	3+	3+	3+	+	1+	-
3	-	-	-	-	-	-	-	-
4	2+	-	-	3+	-	-	1+	-
5	-	-	-	-	-	-	-	1+
6	-	+	1+	+	1+	-	1+	3+
7	2+	-	-	-	-	1+	-	1+
8	-	-	-	-	-	-	1+	3+
9	-	-	-	-	1+	-	-	3+

10	2+	-	1+	-	-	-	1+	3+
11	-	-	1+	3+				
12	-	-	-	1+				

Dip stick test has been performed for these urine samples to test for the presence of Leukocytes, Nitrites, Protein, and Erythrocytes as a sign of infection and to see if there is any correlation between infection status and levels of sTLR4 in urine (Table 4).

From the table, we can see that samples from some patients had higher sTLR4 values but were not dipstick-positive, while for some other patients' samples were positive for dipstick test but sTLR4 values were very low or below detectable range indicating that there is no clear correlation between dipstick positivity and sTLR4 values.

4. Discussion

Globally, more than 2.5 million people suffer from MS, a chronic neurologic condition resulting from inflammatory demyelination of the axons. As a typical autoimmune disease, there is no known single cause of MS; it is thought to be a combination of genetic susceptibility and other triggers such as viral infection, metabolism or other environmental signals, that lead to persistent inflammation in the CNS [21].

TLR signalling plays an important role in the pathogenesis of autoimmune diseases, including MS and also in the progression of MS. TLRs including TLR2 and TLR4 are involved in the production of pro-inflammatory cytokines such as IL-17 and IFN-γ in response to PAMPS and DAMPS during MS [23]. The TLR system must be regulated tightly both in pathologic and physiologic states, otherwise it could lead to the development of autoimmunity [29]. Even though the clinical diagnosis of MS is facilitated by magnetic resonance imaging and laboratory techniques, the detection of reliable biomarkers that would help in the assessment of disease activity and response to treatment would be very useful. Soluble TLRs (sTLR) were reported to occur naturally, often being regulated in response to inflammation as a negative feedback mechanism to inflammation and thus sTLRs could be a good candidate as a biomarker of disease activity in chronic and relapsing inflammation. With some studies showing an association of TLR2 and TLR4 in MS, it is important to verify the role of their soluble forms, sTLR2 and sTLR4, which are easier to detect and quantify.

Here we report the first investigation on the levels of sTLR2, and 4 in serum and urine samples of MS patients using ELISA assay. In comparison to healthy controls, our data showed that the level of sTLR2 in serum was significantly higher in patients during relapse compared to the healthy controls. This may suggest that higher levels of sTLR2 reflect an active state of the immune system during an MS relapse. Similarly, in other pathological states, sTLR2 and 4 were reported to be released into the CSF during aneurysm rupture in subarachnoid haemorrhage (SAH) patients [16]. On the contrary, another study demonstrated that the levels of sTLR2, which acts as endogenous negative regulator of TLR2 signalling, was reduced in serum systemic lupus erythematosus (SLE) patients [29]. The result suggests that TLR2 signalling may be involved in SLE disease course [29].

On the other hand, there were no significant levels of sTLR4 found in the serum of relapse phase patients compared with healthy controls. Also from our study, serum levels of sTLR2 and 4 were not significantly different between relapse and remission states (*P* value>0.99). Comparing healthy controls to remission state in MS, there was also no significant difference in serum concentration of sTLR2 and 4 (*P* value=0.105). This observation is in agreement with a study which showed that the levels of sTLR2 and 4 in CSF samples from 18 patients with acute hydrocephalus following aneurysmal subarachnoid haemorrhage, did not differ significantly compared to control subjects by using ELISA assay [16]. In this study, there were no significant difference in sTLR2 and 4 between paired relapse and remission samples in all patients from both serum and urine. In another study, which made a similar observation where urine TLR4 levels in patients with compensated cirrhosis were found to be lower than in healthy volunteers [28].

Infections are considered to be involved in the susceptibility and clinical course of MS [30]. Acute relapses may be exacerbated by infection, most commonly in the form of urinary tract infection (UTI), or an upper respiratory tract infection (URTI) [30]. We therefore hypothesised that relapse samples could be associated with UTIs. Dipstick test was used in this experiment to test Leukocytes, Nitrites, Protein, and Erythrocytes (Table 3, and 4) which could be considered as a preliminary infection marker. A patient with high levels of sTLR2 gave positive results after the dipstick test for all the four parameters, indicating that patients with MS during relapse could be more susceptible to UTI, even in the absence of symptoms [30]. For example, a clinical audit found that out of 118 patients, 21 patients during relapse were tested positive in urine dipsticks for leucocytes, nitrites, protein, and blood [30]. It has been found in patients with

infection that their sTLR2 and sTLR4 circulating concentrations increased significantly compared with patients with non-infectious inflammation [12]. However, for sTLR4 levels in the urine, there was no correlation with dipstick results, suggesting that sTLR4 levels in urine might not be associated with urinary tract infections.

5. Conclusion

In conclusion, we have shown that sTLR2 level in sera of MS relapse patients was raised compared to healthy controls. In addition, our work indicates that sTLR2 concentration markedly increased in relapsed patients with UTIs unlike in patients with non-infectious inflammation. Although the amount of sTLR2 and sTLR4 in most cases were not significantly different and also below the level of detection in both relapse and remission patients as well as healthy controls, together, these results at least showed the presence of detectable levels of sTLR2 and 4 in MS patients' serum and urine. As this study was limited to a small number of samples, further work with a large cohort of MS patients and healthy individuals are thus important to reach a concrete conclusion about the value of sTLR2 and 4 as a potential biomarker of disease activity. It is also important to see if the level of sTLR2 and 4 has any correlation with other inflammatory markers like C-reactive protein (CRP) levels, IFN-γ or TNF-α expression or with the infection status of the patients, using other sensitive approach such as quantifying mRNA expression of the receptors

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict-of-interest to be disclosed.

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